Activation of PKC-δ in HTLV-1-infected T cells

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Abstract. Protein kinase C (PKC)-δ is a member of the PKC family. It has been implicated in tumor suppression as well as survival of various cancers. The aggressive malignancy of T lymphocytes known as adult T-cell leukemia (ATL) is associated with human T-cell leukemia virus type 1 (HTLV-1) infection. In this study, we show that HTLV-1-infected T cells are characterized by phosphorylation and nuclear translocation of PKC-δ. Expression of HTLV-1 regulatory protein Tax increased PKC-δ phosphorylation. Blockade of PKC-δ by rottlerin suppressed PKC-δ phosphorylation and inhibited cell viability in HTLV-1-infected T-cell lines and primary ATL cells. Rottlerin induced cell cycle arrest at the G_1 phase and caspase-mediated apoptosis of HTLV-1-infected T cells. Rottlerin downregulated the expression of proteins involved in G_1/S cell cycle transition, cyclin D2, CDK4 and 6, and c-Myc, resulting in dephosphorylation of retinoblastoma protein (pRb). Furthermore, rottlerin reduced the expression of important anti-apoptotic proteins (e.g., survivin, XIAP, Bcl-x_L and c-FLIP) and Bcl-2 phosphorylation, and activated the pro-apoptotic protein Bax. Our results showed that permanent activation of nuclear factor-κB (NF-κB) by HTLV-1 Tax allows infected cells to escape cell cycle arrest and apoptosis and that PKC-δ mediates Tax-induced activation of NF-κB. Based on these findings, new therapies designed to target PKC-δ could be potentially useful in the treatment of ATL.

Introduction

Adult T-cell leukemia (ATL) is an aggressive malignancy of $CD4^+$ T cells caused by the retrovirus, human T-cell leukemia virus type 1 (HTLV-1) (1-3). ATL is associated with poor prognosis mainly due to resistance to conventional chemotherapies, hypercalcemia and frequent opportunistic infections due to the associated severe immunosuppression (4). In addition to the classical structural genes required for retroviral replication, the HTLV-1 genome encodes the viral transcriptional activator Tax. The oncogenic potential of Tax has been extensively studied. Especially, permanent activation of the nuclear factor-κB (NF-κB) pathway by Tax is a key event in the process of HTLV-1-induced T-cell immortalization and leukemogenesis (5,6). In the cell nucleus, NF-κB binds promoter DNA elements to initiate or enhance transcription of the genes involved in T-cell survival and cell cycle progression (5,6). Understanding cell survival pathways should enhance the design of new therapeutic approaches against ATL.

The protein kinase C (PKC) family is subdivided into three categories: conventional, novel and atypical PKCs (7). PKC-δ, a novel PKC, plays a crucial role in the cellular response to genotoxic stress. It generally functions as a pro-apoptotic protein during DNA damage-induced apoptosis (8). In contrast, it can act as an anti-apoptotic protein during receptor-initiated cell death (8). PKC-δ promotes cell survival through several well-known pro-survival pathways, including NF-κB, Akt and ERK (8). Thus, PKC-δ has numerous pleiotropic effects and has been implicated in tumor suppression as well as survival of various cancers (8).

The present study demonstrates the constitutive activation of PKC-δ in HTLV-1-infected T-cell lines and freshly isolated ATL cells, and that inhibition of PKC-δ induces cell cycle arrest at the G_1 phase and apoptosis. The results also demonstrate that PKC-δ pathway plays an important role in Tax-mediated NF-κB activation.

Materials and methods

Cells. The HTLV-1-infected MT-2 and -4, C5/MJ, SLB-1, HUT-102, MT-1, TL-OmI and ED-40515(-) T-cell lines, and the negative control uninfected human leukemia Jurkat, MOLT-4 and CCRF-CEM T-cell lines, were grown in Roswell Park Memorial Institute-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics. JPX-9 cells are derivatives of Jurkat with *Tax* gene, which is stably integrated under the control of a metallothionein promoter (9). To induce Tax expression, JPX-9 cells were

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cultured in the presence of 20 μM $CdCl_2$. After obtaining informed consent, blood samples were obtained from a healthy volunteer, six patients with acute type ATL and a patient with chronic type ATL. Peripheral blood mononuclear cells (PBMC) were isolated from the heparinized blood samples by centrifugation over a Ficoll-Paque layer (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The diagnosis of ATL was based on clinical features, hematological findings and the presence of anti-HTLV-1 antibodies in the serum.

Reagents and antibodies. The selective PKC-δ inhibitor, rottlerin, and the general PKC inhibitor, bisindolylmaleimide, were purchased from Calbiochem (Darmstadt, Germany). Antibodies specific for PKC-δ, phospho-PKC-δ (Tyr311), cleaved caspase-3, -8 and -9, cleaved poly(ADP-ribose) polymerase (PARP), survivin, Bak, Bcl-x_L, phospho-Bcl-2 (Ser70), and phospho-IκBα (Ser32 and 36) were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against c-IAP2, cyclin D2 and IκBα were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against XIAP and phospho-retinoblastoma protein (pRb) (Ser780) were obtained from Medical & Biological Laboratories, Co., Ltd. (Aichi, Japan). Antibodies against Bax, Bcl-2, c-FLIP, CDK4 and 6, c-Myc and actin were purchased from Neomarkers, Inc. (Fremont, CA, USA). An antibody against active form-specific Bax was purchased from BD Transduction Laboratories (San Jose, CA, USA). Mouse monoclonal antibody to Tax, Lt-4, was described previously (10).

Immunoblotting analysis. To investigate cell proteins by western blot analysis, cells were lysed in a lysis buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate, 10% glycerol, 6% 2-mercaptoethanol and 0.01% bromophenol blue. Lysates were centrifuged and supernatants were collected. Total protein concentration for each sample was determined by the Bradford assay (Bio-Rad, Hercules, CA, USA). Total extracts (20 μg/lane) were subjected to sodium dodecyl sulfate-polyacrylamide gels and western blot analysis was performed on polyvinylidene difluoride membranes. Protein expression was analyzed by immunoblotting using the specific antibodies. The bands were visualized with the enhanced chemiluminescence system (Amersham Biosciences Corp, Piscataway, NJ, USA).

Immunofluorescence staining. Cells were fixed with 4% paraformaldehyde for 20 min at 37˚C. Fixed cells were washed twice with phosphate-buffered saline (PBS) containing 7% of FBS and permeabilized with PBS containing 0.1% Triton X-100 for 10 min at room temperature. In the next step, the cells were washed once with PBS containing 7% of FBS and resuspended in PBS/7% FBS containing antibody against PKC-δ or phospho-PKC-δ (Tyr311) for 20 min at room temperature. Then, the cells were washed twice with PBS/7% FBS and resuspended in PBS/7% FBS containing Alexa Fluor 488-labeled goat anti-rabbit IgG (Invitrogen Life Technologies, Carlsbad, CA, USA) for 20 min at room temperature. The nuclei were stained with Hoechst 33342 (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Finally, the cells were washed twice with PBS and examined under a Leica DMI6000 microscope (Leica Microsystems, Wetzlar, Germany). Mounted coverslips were imaged through a 63x oil immersion lens (NA1.4) on a Leica TCS confocal system.

Cell viability and proliferation assays. Cell viability and proliferation were assessed by measuring mitochondrial-dependent conversion of water-soluble tetrazolium (WST)-8 (Nacalai Tesque, Inc., Kyoto, Japan) to a colored formazan product. Cell lines or PBMC were seeded in 96-well plates and then treated with rottlerin or bisindolylmaleimide for 48 or 24 h, respectively. This was followed by the addition of a solution containing WST-8 to the cells for 4-6 h at 37˚C and the viable growing cells were estimated by monitoring the adsorption of the product at 450 nm. All experiments were performed in triplicates.

Analysis of cell death. Apoptosis was assessed by the APO2.7 assay. Cells were seeded in culture plates then treated with rottlerin (20 μM) for 48 h, followed by analysis by flow cytometry after staining with phycoerythrin-conjugated APO2.7 antibody (Beckman Coulter, Marseille, France), which specifically detects 7A6, a 38-kDa mitochondrial membrane antigen expressed during apoptosis (11). In addition, apoptosis was also assessed by monitoring cleavage of caspase-3, -8 and -9, as well as PARP by western blot analysis.

In vitro measurement of caspase activity. Caspase activity was measured using Colorimetric Caspase Assay kits (Medical & Biological Laboratories, Co., Ltd.). Briefly, cell extracts were recovered using the cell lysis buffer supplied with the kit and assessed for caspase-3, -8 and -9 activities using colorimetric probes. The assay kits are based on detection of chromophore ρ-nitroanilide after cleavage from caspase-specific labeled substrates. Colorimetric readings were performed in an automated microplate reader.

Cell cycle analysis. Cells were harvested and the CycleTEST Plus DNA Reagent kit (Becton-Dickinson Immunocytometry Systems, San Jose, CA, USA) was used for analysis of changes in the cell cycle. Cell suspensions were analyzed by flow cytometry. The MultiCycle software calculated the percentage of cells in each cell cycle phase.

Immunohistochemical analysis. Lymph node biopsies were obtained from patients with ATL. Phospho-PKC-δ immunohistochemistry was performed using an anti-phospho-PKC-δ (Tyr311) antibody after pre-treatment of the deparafinized tissue sections with ready-to-use proteinase K (Dako, Carpinteria, CA, USA). The sections were counterstained with methyl green, hydrated in ethanol, cleaned in xylene, and mounted. The stained cells were examined under a light microscope (Axioskop 2 Plus) with an Achroplan 40x/0.65 lens (both from Zeiss, Jena, Germany). Images were acquired with an AxioCam MRc camera and AxioVision 4.7 software (Zeiss). A signed consent form was obtained from each tissue donor.

Plasmids and transfection. The PKC-δ constructs of pGFP-PKC-δ (δWT), in which the GFP tag was placed on the C-terminus of the kinase, pGFP-PKC-δ^{K376R} (δKN), pGFP-PKC-δ^{D327A} (δCM), pGFP-NLS PKC-δ (NLS-δWT),

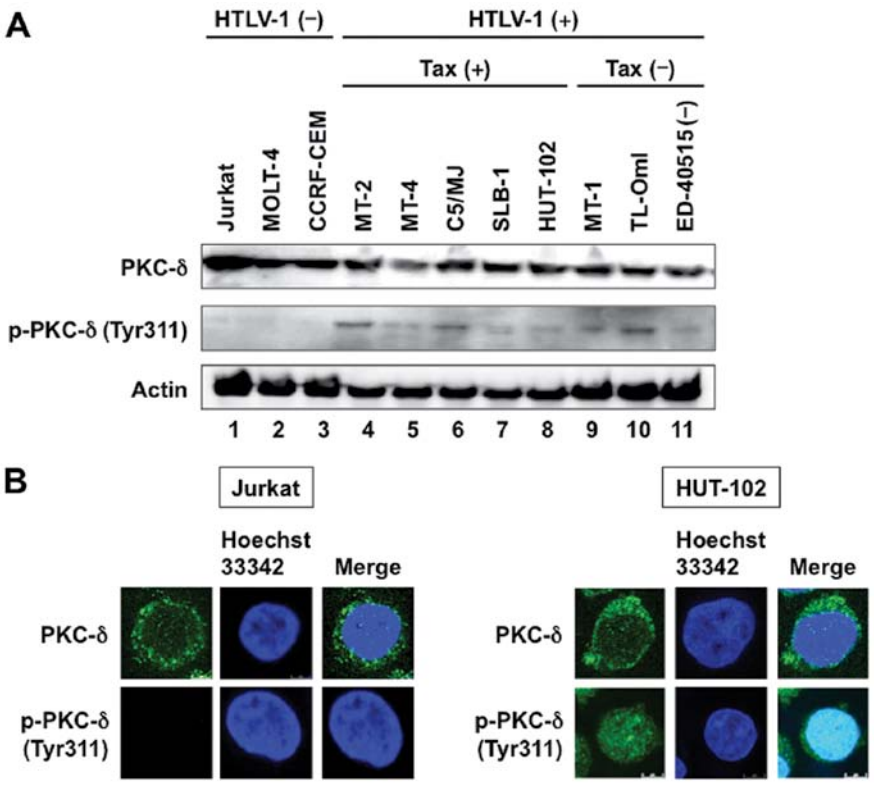

Figure 1. Phosphorylation of protein kinase C (PKC)-δ in HTLV-1-infected T-cell lines. (A) Western blot analysis of phosphorylated and total PKC-δ in uninfected T-cell lines (lanes 1-3), HTLV-1-transformed T-cell lines (lanes 4-8) and adult T-cell leukemia (ATL)-derived T-cell lines (lanes 9-11). Membranes were reprobed with the anti-actin antibody as a loading control. (B) Phosphorylated PKC-δ is localized in the nuclei of HTLV-1-infected T cells. Jurkat and HUT-102 cells were stained with an antibody specific for PKC-δ or phospho-PKC-δ (green) with Hoechst 33342 (blue) to identify the nuclei. The cells were visualized by confocal microscopy.

pGFP-NLS PKC-δ^{K376R} (NLS-δKN) and pGFP-NLS PKC-δ^{D327A} (NLS-δCM) were previously described (12,13). These mouse wild-type PKC-δ and its mutants were cloned into pEGFP-N1 (Clontech Laboratories, Palo Alto, CA, USA). The K376→R mutation in kinase-negative residues in the ATP-binding site and the protein has been shown to function as an isoform-specific dominant inhibitory kinase (14). The D327→A mutation in the caspase cleavage site resulted in loss of caspase cleavage (12). To drive nuclear import, an SV40 nuclear localization signal (NLS) was fused to the N-terminus of PKC-δ (NLS-δWT) (13). A reporter plasmid, expressing luciferase through a minimal promoter linked to five copies of the typical NF-κB responsive element from the *interleukin-2 receptor α chain* gene (κB-LUC), was used to measure the NF-κB transcriptional competence (15). Plasmid expressing the HTLV-1 Tax through β-actin promoter has been described (16). 293T cells were transfected by the calcium phosphate DNA co-precipitation method. Each transfection included the phRL-TK plasmid (Promega Corp., Madison, WI, USA) as an internal control for variation in the transfection efficiency. Total DNA was completed to 2,042 ng in all samples with an empty plasmid. The luciferase activity was determined by the Dual-Luciferase Reporter system (Promega Corp.) using the protocol supplied by the manufacturer.

Preparation of nuclear extracts and EMSA. Nuclear proteins were extracted and transcription factors bound to specific DNA sequences were examined by electrophoretic mobility shift assay (EMSA), as described previously (17). The top strand sequences of the oligonucleotide probes were as follows: for a typical NF-κB element from the *interleukin-2 receptor α chain* gene, 5'-GATCCGGCAGGGGAATCTCCCTCTC-3'; and for the consensus sequence of the octamer binding motif, 5'-GATCTGTCGAATGCAAATCACTAGAA-3'. The latter was used to identify specific binding of the transcription factor Oct-1, which regulates the transcription of a number of so-called housekeeping genes. The above underlined sequences represent the NF-κB and Oct-1 binding sites, respectively.

Statistical analysis. Data were expressed as mean ± SD, and statistical differences in parameters between groups were examined using the Student's t test. $P<0.05$ denoted the presence of a statistically significant difference.

Results

Constitutive phosphorylation of PKC-δ in HTLV-1-infected T-cell lines. In contrast to serine/threonine phosphorylation sites, tyrosine phosphorylation is a relatively specific regulatory mechanism for PKC-δ and not a common regulatory mechanism for the entire family of PKC enzymes. Phosphorylation of Tyr311 in the hinge region of PKC-δ has been reported to initiate a series of phosphorylation reactions on other tyrosines that play important roles in the regulation of its activity (18). We initially examined the phosphorylation of PKC-δ at Tyr311 in HTLV-1-infected and -uninfected

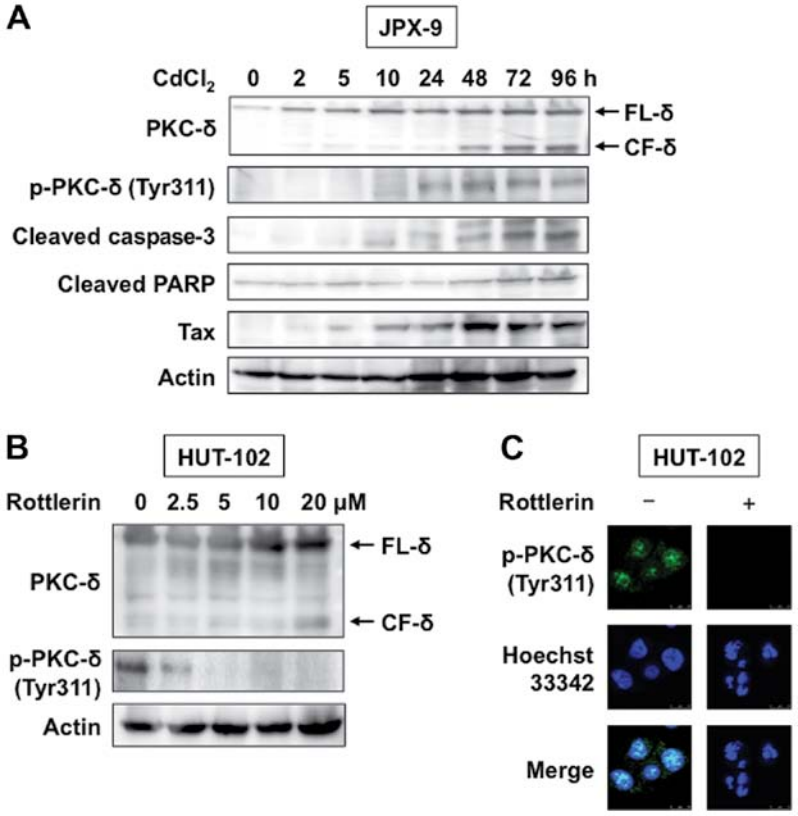

Figure 2. HTLV-1 Tax induced phosphorylation and cleavage of PKC-δ, and inhibition of protein kinase C (PKC)-δ induced chromatin condensation and nuclear fragmentation in HTLV-1-infected T cells. (A) Tax induced apoptosis, and phosphorylation and cleavage of PKC-δ. JPX-9 cells were treated with $CdCl_2$ (20 μM) for the indicated time intervals then harvested and whole cell lysates were prepared. The protein content of 20 μg was subjected to immunoblot analysis using specific antibodies. The positions of the full-length (FL-δ) and the catalytic fragment (CF-δ) are indicated. (B) Rottlerin treatment blocked PKC-δ phosphorylation and cleaved PKC-δ in HTLV-1-infected T cells. HUT-102 cells were incubated with the indicated concentrations of rottlerin for 48 h. Whole cell lysates were examined. (C) Rottlerin induces PKC-δ dephosphorylation, chromatin condensation and nuclear fragmentation. HUT-102 cells were treated with or without 20 μM rottlerin for 48 h. The cells were stained with an antibody specific for phospho-PKC-δ (green) and Hoechst 33342 (blue) to identify the nuclei. The cells were visualized by confocal microscopy.

T-cell lines. HTLV-1-transformed T-cell lines, including MT-2 and -4, C5/MJ, SLB-1 and HUT-102, constitutively expressed Tax mRNA. In contrast, ATL-derived T-cell lines, including MT-1, TL-OmI and ED-40515(-), did not express Tax (19). Western blot analysis showed ubiquitous expression of PKC-δ in all human T-cell lines (Fig. 1A, top panel). All eight HTLV-1-infected T-cell lines constitutively expressed the phosphorylated form of PKC-δ, although the phosphorylated level varied widely. On the other hand, the phosphorylated form of PKC-δ was not detected in three uninfected T-cell lines (Fig. 1A, middle panel).

In the next step, immunofluorescent staining was used to map the site(s) of PKC-δ. In uninfected Jurkat cells, PKC-δ was localized primarily in the cytoplasm and the phosphorylated form of PKC-δ could not be detected (Fig. 1B, left panels). PKC-δ was also localized primarily in the cytoplasm in HTLV-1-infected HUT-102 cells. However, the nuclear phosphorylated form of PKC-δ was detected in HUT-102 cells (Fig. 1B, right panels).

Tax induces PKC-δ phosphorylation. Next, we analyzed the role of Tax in the regulation of PKC-δ phosphorylation. For this purpose, JPX-9 cells transfected with the *Tax* gene, regulated by the inducible metallothionein promoter, were used for Tax-function analysis, as described previously (9). Treatment of JPX-9 cells with $CdCl_2$ induced significant expression of Tax protein (Fig. 2A, panel 5). Phosphorylation of PKC-δ was also induced in $CdCl_2$-treated JPX-9 cells (Fig. 2A, second panel). Various apoptotic agents can cleave PKC-δ from caspases, resulting in the production of a 40-kDa catalytic fragment (CF-δ) (12). Tax is reported to cause apoptosis of JPX-9 cells (20), although it is believed to play a significant role in transforming host cells. To characterize and confirm that death of JPX-9 cells was due to apoptosis, we used western blot analysis to investigate cleavage of caspase-3 and its specific substrate PARP. Consistent with apoptotic death, we observed processing of caspase-3 and PARP in JPX-9 cells treated with $CdCl_2$ (Fig. 2A, panels 3 and 4). Treatment with $CdCl_2$ induced Tax-mediated apoptosis, resulting in caspase cleavage of PKC-δ (Fig. 2A, top panel).

Inhibition of PKC-δ by rottlerin decreases cell viability and induces apoptosis of HTLV-1-infected T cells. We next analyzed the effects of rottlerin, a PKC-δ inhibitor, on HTLV-1-infected HUT-102 cells. Western blot analysis and immunofluorescent staining showed that rottlerin reduced tyrosine phosphorylation of PKC-δ (Fig. 2B and C). Furthermore, rottlerin induced PKC-δ cleavage as demonstrated by western blot analysis, as well as chromatin condensation and nuclear fragmentation, as demonstrated using Hoechst 33342

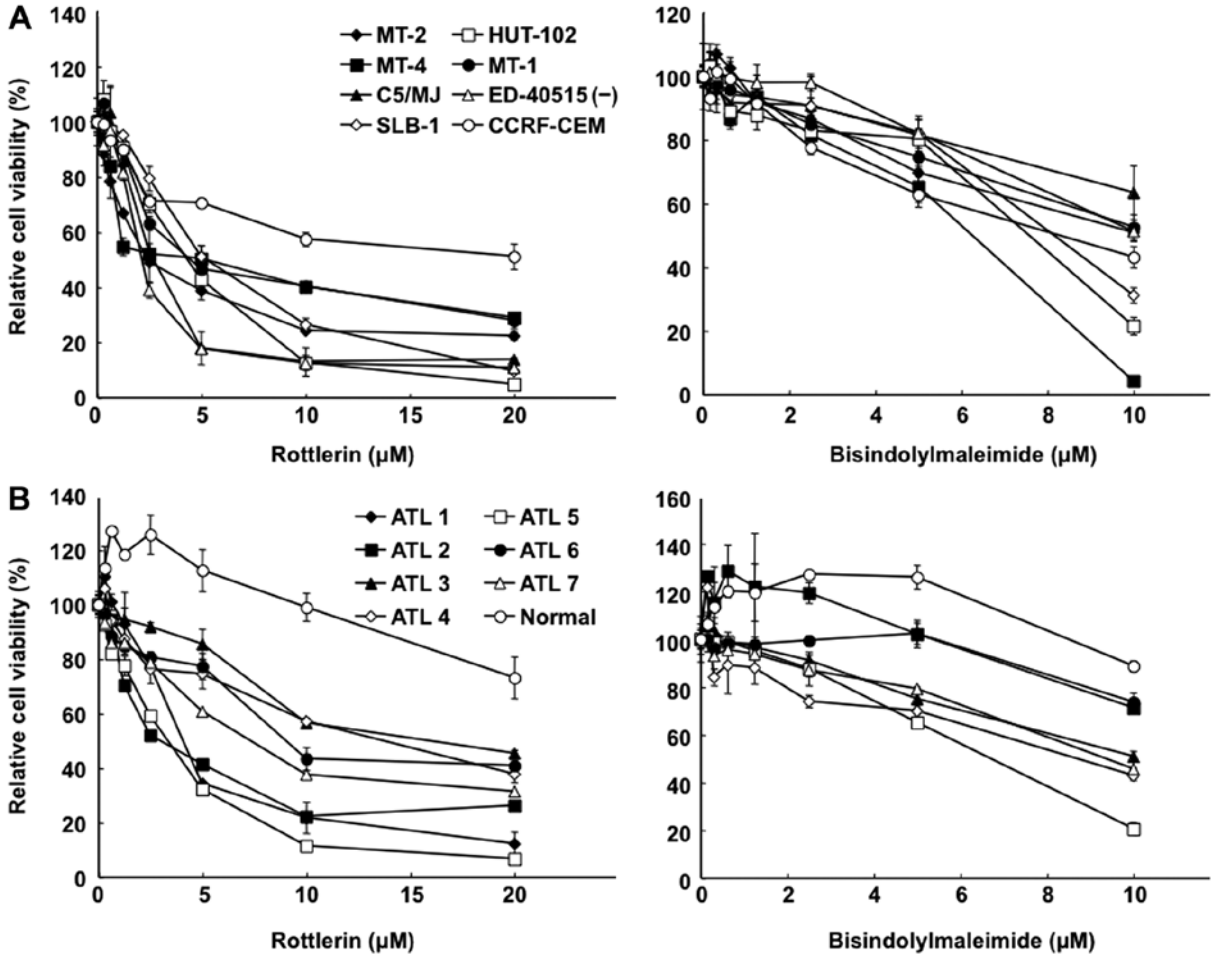

Figure 3. Effects of protein kinase C (PKC)-δ inhibition on viability of various human T-cell lines and peripheral blood mononuclear cells (PBMC). (A) Human T-cell lines and (B) PBMC were incubated with various concentrations of rottlerin or bisindolylmaleimide. After 24 h (PBMC) or 48 h (cell lines), viability was determined in triplicate cultures using the water-soluble tetrazolium (WST)-8 assay. The results are expressed as mean ± SD percentages of the control (n=3).

staining (Fig. 2B and C). In the next step, we compared the effects of PKC-δ-specific inhibitor rottlerin and general PKC inhibitor bisindolylmaleimide on the viability of HTLV-1-infected T-cell lines. Rottlerin inhibited the viability of HTLV-1-infected T-cell lines, MT-2 and -4, C5/MJ, SLB-1, HUT-102, MT-1 and ED-40515(-) in a dose-dependent manner. In contrast to HTLV-1-infected T-cell lines, uninfected T-cell line CCRF-CEM was less susceptible to rottlerin (Fig. 3A, left panel). While bisindolylmaleimide reduced cell viability of HTLV-1-infected T-cell lines, its effect was less pronounced than that of rottlerin. Furthermore, there was no difference in the susceptibility of HTLV-1-infected and -uninfected T-cell lines to bisindolylmaleimide (Fig. 3A, right panel).

We also investigated the effects of rottlerin and bisindolylmaleimide on peripheral ATL cells freshly isolated from seven patients with ATL. Rottlerin reduced ATL cell survival but not that of PBMC obtained from a healthy donor (Fig. 3B, left panel). Bisindolylmaleimide also reduced cell viability though its effect was milder than that of rottlerin (Fig. 3B, right panel).

Since rottlerin reduced the viability of HTLV-1-infected T cells, we next assessed the effects of rottlerin on apoptosis. Inhibition of PKC-δ by 20 μM rottlerin increased the number of apoptotic cells as measured by APO2.7 staining (Fig. 4A). Interestingly, HTLV-1-infected T-cell lines were more susceptible to rottlerin than uninfected CCRF-CEM cells (Fig. 4A). Next, we studied the role of caspases in this process by determining cleavage of endogenous caspases. Western blot analysis demonstrated increased levels of activated cleaved forms of caspase-3, -8 and -9, as well as PARP, and that such increases were rottlerin dose-dependent (Fig. 4B). Immunoblotting allowed us to examine the processing of caspases, but did not indicate whether the cleavage products were enzymatically active. Therefore, we used colorimetric assays to determine caspase-3, -8 and -9 activities based on cleavage of caspase-specific-labeled substrates. As shown in Fig. 4C, rottlerin activated caspase-3, -8 and -9 in HUT-102 cells. These results confirmed that caspase activation mediates rottlerin-induced apoptosis of HTLV-1-infected T-cell lines.

As shown in Fig. 4D, rottlerin inhibited PKC-δ in freshly isolated primary ATL cells, and resulted in cleavage of caspase-3, -8 and -9, as well as PARP in these cells. Furthermore, immunohistochemical staining showed nuclear staining for phosphorylated PKC-δ (Tyr311) in ATL cells found in lymph nodes (Fig. 4E).

Rottlerin induces cell cycle block in HTLV-1-infected T cells. Cell cycle analysis showed that rottlerin significantly increased the population of HTLV-1-infected T cells at the G_1 phase of

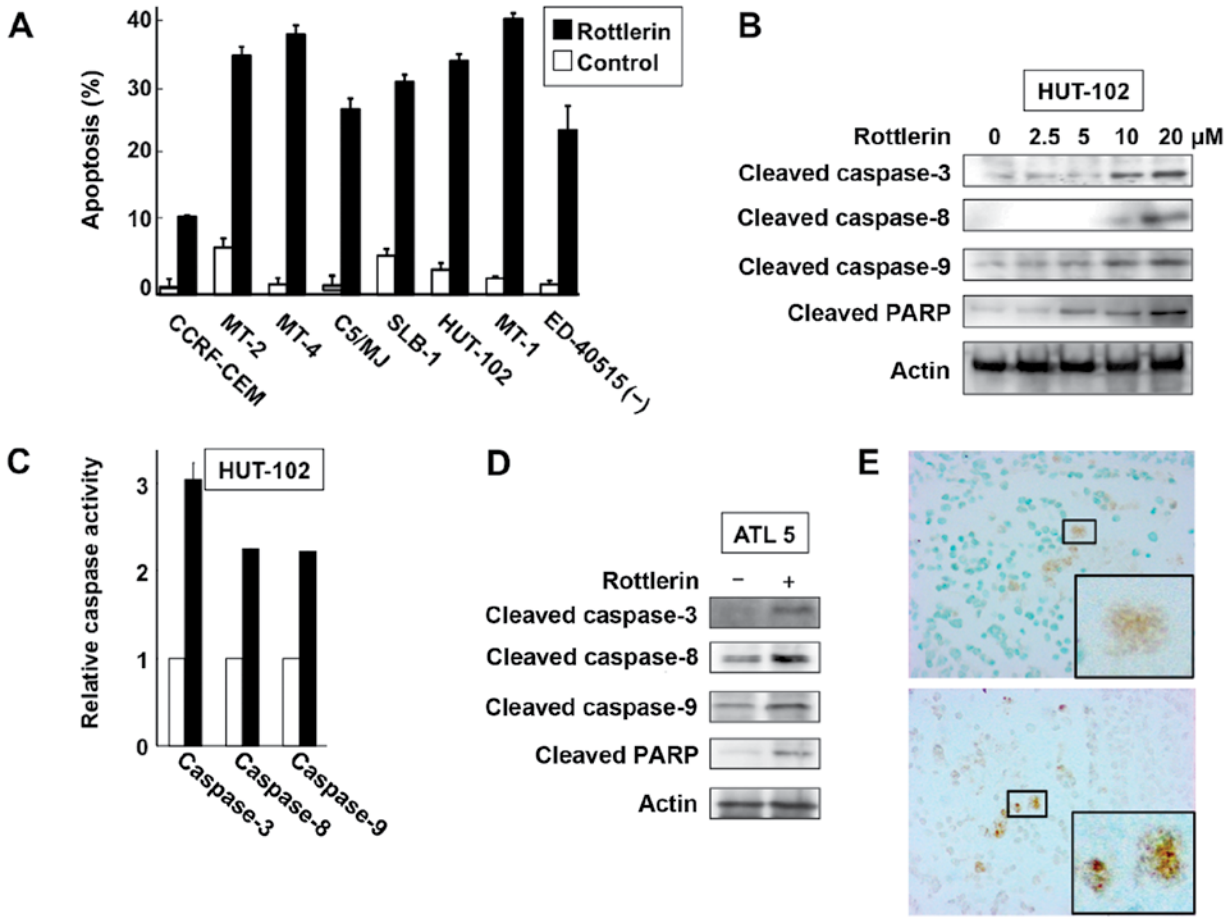

Figure 4. Rottlerin-induced apoptosis involves activation of caspase-3, -8 and -9. (A) Flow cytometric analysis of APO2.7 reactive cells. Human T-cell lines were treated with or without 20 μM of rottlerin for 48 h. The cells were analyzed by flow cytometry after staining with phycoerythrin-conjugated APO2.7 antibody. Data are mean ± SD percentages of apoptotic cells for both untreated (open bars) and rottlerin-treated (solid bars) cells (n=3). (B) Immunoblot analysis of cleaved caspase-3, -8 and -9, as well as poly(ADP-ribose) polymerase (PARP). HUT-102 cells were treated with the indicated concentrations of rottlerin for 48 h. Samples of 20 μg of whole cell lysates were examined by immunoblotting. The immunoblot of actin served as a control. (C) Rottlerin-induced apoptosis is caspase-dependent, based on treatment of HUT-102 cells with or without 20 μM of rottlerin. After 48 h, cell lysates were prepared and incubated with the labeled caspase substrates, and caspase activity was measured using an automated microplate reader. Caspase activity is expressed relative to untreated cells, which was assigned an arbitrary value of 1. Data are mean ± SD (n=3). (D) Immunoblot analysis of cleaved caspase-3, -8 and -9, as well as PARP. Peripheral blood mononuclear cells (PBMC) from a patient with adult T-cell leukemia (ATL) were treated with or without 10 μM of rottlerin for 24 h. Samples of 20 μg of whole cell lysates were examined by immunoblotting. (E) Immunohistochemical staining of phosphorylated protein kinase C (PKC)-δ in ATL lymph nodes. Tissue sections from ATL lymph nodes were stained with anti-phospho PKC-δ antibody and counterstained with methyl green. Low-power images of representative lymph nodes from patients with ATL (original magnification, x400). The inset represents higher magnification of the small boxed region (original magnification, x1,200).

the cell cycle, and at the same time it decreased the population of cells at the S phase. However, rottlerin had no effect on the percentage of CCRF-CEM cells in the G_1 and S phases, compared to the control (Fig. 5). These results suggest that PKC-δ accelerates the cell cycle in HTLV-1-infected T cells, i.e., inhibition of PKC-δ causes cell cycle arrest at the G_1 phase.

Inhibition of PKC-δ results in downregulation of anti-apoptotic proteins and activation of pro-apoptotic protein. High levels of anti-apoptotic proteins are commonly found in HTLV-1-infected T cells, suggesting their potential involvement in the pathophysiology of the disease. Therefore, we studied the expression of anti- and pro-apoptotic proteins in HTLV-1-infected T cells cultured with rottlerin. As shown in Fig. 6 (left panels), HUT-102 cells expressed high levels of survivin, c-IAP2, XIAP, Bcl-x_L, Bcl-2 and c-FLIP. Incubation of these cells with rottlerin induced downregulation of survivin, XIAP, Bcl-x_L and c-FLIP. Although rottlerin had no effect on the level of Bcl-2, it significantly reduced phosphorylation of Bcl-2 at Ser70 when used at concentration of 2.5 μM and completely abolished the phosphorylation at 20 μM. Bcl-2 phosphorylation at Ser70 is necessary for its full and potent anti-apoptosis function (21).

The pro-apoptotic Bcl-2 family proteins, such as Bax and Bak, act on the mitochondria and counterbalance Bcl-2. The protein expression of Bax and Bak remained stable regardless of PKC-δ inhibition. Bax-mediated cell death occurs via well-controlled steps, including a conformational change that facilitates the dimerization and translocation of Bax to the mitochondrial outer membrane (22,23). For this reason, we also tested the involvement of Bax activation in apoptosis. Conformational changes in Bax can be evaluated using conformation-specific anti-Bax antibodies (24). In the present study, immunoblot analysis using an antibody specific for the active form of Bax (clone 3) demonstrated that rottlerin changed Bax conformation in a dose-dependent manner.

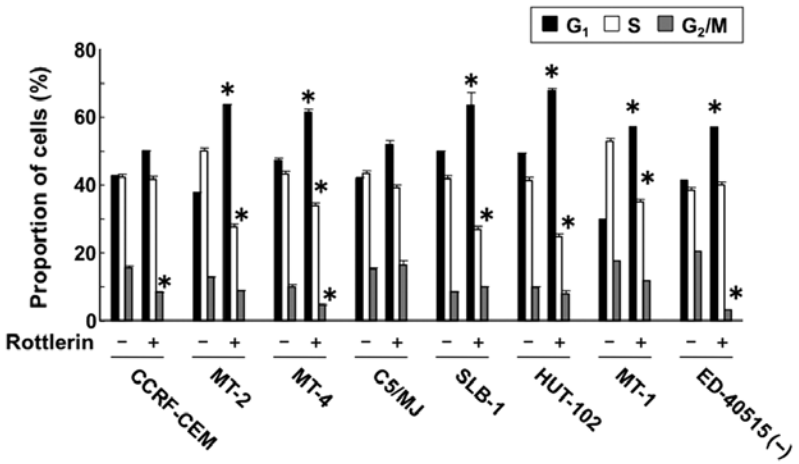

Figure 5. Rottlerin increases G_1 population in HTLV-1-infected T-cell lines. The indicated human T-cell lines were treated with or without 20 μM of rottlerin for 24 h. Cells were harvested and stained with propidium iodide. Cell cycle distribution was analyzed using flow cytometry. Data are expressed as mean ± SD percentage of cells at various phases of the cell cycle (n=3). *P<0.05 compared with the control.

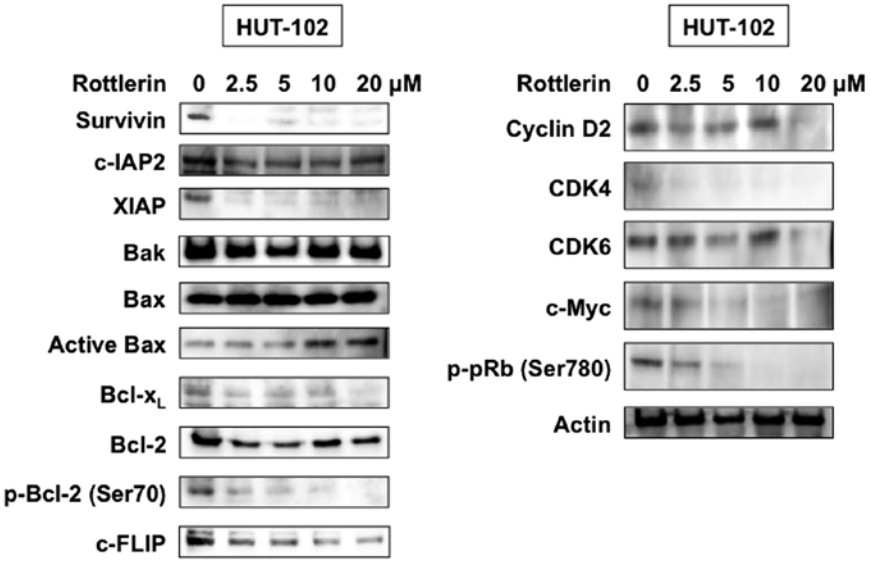

Figure 6. Blockade of protein kinase C (PKC)-δ modulates the expression of pro- and anti-apoptotic, as well as cell cycle-regulatory proteins. HUT-102 cells were treated with the indicated concentrations of rottlerin for 48 h, then collected and lysed. Total protein (20 μg) was subjected to immunoblot analysis by using specific antibodies against pro- and anti-apoptotic proteins (left panels), as well as cell cycle-regulatory proteins (right panels).

Inhibition of PKC-δ alters the expression of proteins involved in cell cycle regulation. In order to determine whether the rottlerin-induced cell cycle block is accompanied by changes in proteins involved in cell cycle regulation, we studied the expression of cyclin D2, CDK4 and 6 proteins in HUT-102 cells. Fig. 6 (right panels) shows that rottlerin decreased cyclin D2, CDK4 and 6 protein levels compared to the control. The product of the proto-oncogene c-Myc is a potent activator of cell proliferation. *Cyclin D2* and *CDK4* genes are direct targets of c-Myc (25). CDK4 and 6 are responsible for phosphorylation of pRB, the product of the retinoblastoma tumor suppressor gene (25). Rottlerin significantly reduced c-Myc and the hyperphosphorylated pRb form.

PKC-δ kinase activity is required for Tax-induced NF-κB activation. NF-κB is a target of Tax, and its activation progresses cell cycle and prevents apoptosis through regulation of expression of pro-proliferation and anti-apoptosis genes in HTLV-1-infected T cells (5,6). To determine the involvement of PKC-δ in Tax-induced NF-κB activation, 293T cells were co-transfected with a luciferase reporter plasmid containing five copies of the NF-κB motif (κB-LUC) and expression plasmids for wild-type PKC-δ and its mutants in the presence or absence of Tax expression plasmid. Tax induced κB-LUC activity (Fig. 7A), and the induction was significantly increased by wild-type PKC-δ (δWT), but markedly inhibited by the kinase-dead PKC-δ (δKN). To determine whether nuclear retention of PKC-δ is necessary for Tax-mediated NF-κB activation, 293T cells were co-transfected with NLS-δWT. Fusion of the SV40 NLS to PKC-δ (NLS-δWT) resulted in a much higher κB-LUC activity. In contrast, the kinase-negative construct of NLS-δWT (NLS-δKN) significantly inhibited Tax-mediated κB-LUC activity. As expected, mutation at the caspase cleavage site (δCM and NLS-δCM) did not affect κB-LUC activity by

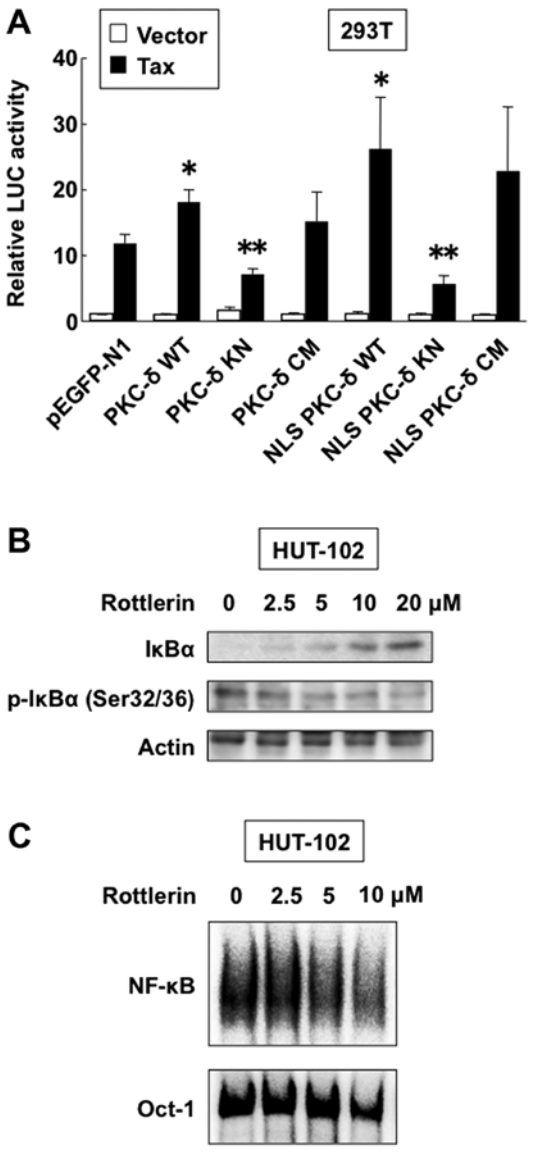

Figure 7. Protein kinase C (PKC)-δ regulates Tax-induced nuclear factor-κB (NF-κB) activation. (A) Effects of PKC-δ constructs on Tax-induced NF-κB activation. The 293T cells were co-transfected with 2 ng of κB-LUC and 2 μg of the indicated PKC-δ constructs in the presence or absence of 20 ng of Tax-expression plasmid. After 24 h, the cells were harvested and the luciferase assay was performed. The activities are expressed relative to that of cells transfected with κB-LUC and empty vectors, which was defined as 1. Data are mean ± SD of three independent experiments (*P<0.05 and **P<0.01 compared with the control cells transfected with Tax-expression plasmid alone). LUC, luciferase. (B) Effect of rottlerin on total and phospho-IκBα levels. HUT-102 cells were treated with the indicated concentrations of rottlerin for 48 h. Whole cell extracts were prepared for measuring the levels of total IκBα or phospho-IκBα by western blot analysis. β-actin was used as a loading control. (C) Inhibition of NF-κB binding activity in HTLV-1-infected T cells. HUT-102 cells were treated with the indicated concentrations of rottlerin for 48 h. Nuclear extracts were examined for NF-κB binding activity by electrophoretic mobility shift assay (EMSA) with a radiolabeled NF-κB specific probe.

δWT and NLS-δWT. These results highlight the importance of active nuclear PKC-δ in Tax-mediated NF-κB activation.

NF-κB activation in HTLV-1-infected T cells is mediated by PKC-δ. Overexpression of a dominant negative form of PKC-δ resulted in significant attenuation of Tax-mediated NF-κB activation. To further delineate the involvement of PKC-δ in NF-κB activation in HTLV-1-infected T cells, HUT-102 cells were treated with rottlerin. Western blot analysis detected phosphorylation and degradation of IκBα in control HUT-102 cells (Fig. 7B). Rottlerin inhibited the phosphorylation and degradation of IκBα. EMSA studies showed that rottlerin inhibited NF-κB binding activity (Fig. 7C). Under the same experimental conditions, rottlerin did not affect Oct-1 binding activity, suggesting the specificity of rottlerin inhibition. Taken together, the above results stress the role of PKC-δ in NF-κB activation in HTLV-1-infected T cells.

Discussion

HTLV-1-infected T cells exhibit constitutive activation of various signaling pathways (26). Such activation plays an important role in apoptosis in ATL. One of the important features of HTLV-1-infected T cells is the high level of NF-κB activity (5,6).

In the present study, we demonstrated constitutive phosphorylation of PKC-δ in HTLV-1-infected T cells and that specific inhibition of this kinase with rottlerin increased apoptosis and induced cell cycle arrest at the G_1 phase in HTLV-1-infected T cells. In contrast, normal PBMC from a healthy donor were resistant to rottlerin-induced cell death. We also demonstrated that shutting down the constitutive activity of PKC-δ resulted in a strong apoptotic response in HTLV-1-infected T cells, primarily by affecting the expression and activity of proteins acting on both the cell membrane and mitochondria. Activation of initiator caspases (caspase-8 and -9) led to the proteolytic activation of downstream effector caspase-3. Two independent pathways, death receptors and the mitochondria, lie upstream of caspase activation. Downregulation of c-FLIP by rottlerin may result in activation of caspase-8, because c-FLIP has anti-apoptotic properties by blocking the activity of FLICE. Anti-apoptotic Bcl-2 family proteins, such as Bcl-2 and Bcl-x_L, disrupt the mitochondrial apoptotic machinery, while the pro-apoptotic member Bax is central to cell apoptosis. Downregulation of Bcl-x_L and phosphorylated Bcl-2 proteins, and activation of Bax may result in the activation of caspase-9. Survivin and XIAP, members of the inhibitor of apoptosis (IAP) family, can directly bind to and suppress several caspases (27). In addition to changes in Bcl-2 family proteins, any decrease in IAP family protein levels may result in the activation of caspase-3 and -9.

The results also showed that rottlerin increased the percentage of HTLV-1-infected T cells in the G_1 phase of the cell cycle. The growth arrest evoked by rottlerin was caused by marked decrease in the amount of cyclin D2, CDK4 and 6, as well as c-Myc, which directly regulate the G_1 to S phase progression of the cell cycle.

The expression of genes that suppress apoptosis (e.g., survivin, XIAP, Bcl-x_L and c-FLIP) or mediate cell proliferation (e.g., cyclin D2, CDK4 and 6, as well as c-Myc) is regulated by NF-κB in HTLV-1-infected T cells (28-34). NF-κB is normally present in the cytoplasm by inhibitory proteins such as IκBα. Induction of NF-κB is associated with phosphorylation and release of IκBα from NF-κB complexes followed by proteolytic degradation. PKC-δ activates NF-κB signaling, and rottlerin can prevent NF-κB activation (35-41). In this regard, the present study showed that rottlerin interfered with the NF-κB activation process by lowering the levels of phospho-IκBα. These findings demonstrate the interfering

action of rottlerin in NF-κB signaling pathway, inhibition of which is fully consistent with the decrease in the above NF-κB-regulated gene products.

HTLV-1 Tax induces NF-κB activation (5,6). The present study provides evidence for the importance of PKC-δ in the NF-κB induction pathway. Overexpression of dominant negative PKC-δ inhibits Tax induction of NF-κB. Furthermore, forced expression of wild-type PKC-δ augmented Tax-induced NF-κB activation, and fusion of the SV40 NLS to PKC-δ resulted in targeting the protein to the nucleus and further increase in Tax-mediated NF-κB activation. Endogenous PKC-δ is located primarily in the cytosol, but phosphorylated PKC-δ is present within the nucleus of HTLV-1-infected T cells and ATL cells invading lymph nodes. Our studies clearly demonstrate that nuclear import of active PKC-δ is required for Tax-mediated NF-κB activation. This conclusion is supported by the findings of previous studies (42), which showed direct interaction of Tax with PKC-δ and Tax-induced phosphorylation of PKC, and that subsequent steps in the PKC cascade seem to have stimulated IκBα phosphorylation.

Phosphorylation of PKC-δ was also observed in ATL-derived T-cell lines that did not express Tax. Similarly, ATL-derived T-cell lines as well as ATL leukemic cells sustain NF-κB activity without Tax (43). These findings suggest that phosphorylated PKC-δ may play an important role in Tax-independent NF-κB activation.

In conclusion, we demonstrated in the present study selective phosphorylation of PKC-δ in HTLV-1-infected T cells. Importantly, we showed that Tax activates NF-κB and this action is mediated through PKC-δ activation. Together with our previous findings that inhibition of NF-κB can induce cell apoptosis (44), our present study further delineates potential upstream regulators of NF-κB, which may play a role in the enhanced survival of HTLV-1-infected T cells. Based on our results, we suggest that rottlerin inhibits the proliferation and survival of HTLV-1-infected T cells through its inhibitory effect on the NF-κB activation process, which diminishes the levels of proliferative and anti-apoptotic NF-κB-regulated gene products. These results could be useful in the design of new PKC-δ-targeting therapies that can potentially improve the prognosis of patients with ATL.

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